



Organic amendments change soil organic C structure and microbial community but not total organic matter on sub-decadal scales

Jiangbing Xu^{a,b}, Sarah S. Roley^{c,1}, Malak M. Tfaily^{d,2}, Rosalie K. Chu^d, James M. Tiedje^{b,*}

^a Institute of Ecology, School of Applied Meteorology, Nanjing University of Information Science and Technology, Nanjing, 210044, China

^b Center for Microbial Ecology, Michigan State University, MI, 48824, United States

^c Great Lakes Bioenergy Research Center, W.K. Kellogg Biological Station, Michigan State University, Hickory Corners, MI, 49060, USA

^d Environmental Molecular Sciences Laboratory, Earth and Biological Sciences Division, Pacific Northwest National Laboratory, Richland, WA, 99354, USA

ARTICLE INFO

Keywords:

Corn stover retention
Rye cover crop
FT-ICR-MS
Phospholipid fatty acid
16S rRNA gene
Bacterial community

ABSTRACT

Organic C has many benefits for soil, but it is depleted by tillage and crop harvest, and especially so for biofuel crops. Accordingly, strategies such as partially retaining stover or planting a cover crop can help ameliorate the negative effect of C removal. We used a long-term field experiment to study the impacts of stover retention and planting a cover crop on soil organic matter (SOM), its extractable components, and the soil microbial community. SOM chemical composition characterization was determined by electrospray ionization (ESI) coupled with Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) in sequential water, methanol (MeOH), and chloroform (CHCl₃) extracts. The characteristics of the soil bacterial community were measured by phospholipid fatty acid (PLFA), real-time quantitative PCR, and 16S rRNA gene sequence. The variations in total SOM content, total microbial biomass, and bacterial population were slight among treatments, but SOM chemical compounds, arbuscular mycorrhizal fungi (AMF) biomass, and bacterial structure changed significantly, and especially so in the coupled application of stover retention and cover crop. Specifically, stover retention enriched more lignin-like compounds in soil, whereas cover crop enriched more condensed hydrocarbons, and had more compounds with an aromaticity index (AI) > 0.5. The bacterial community was not altered by the cover crop, but the corn stover retention increased the relative abundances of Myxococcales (Deltaproteobacteria) and decreased that of Actinobacteria. Redundancy analysis (RDA) further revealed that the bacterial community in the stover treatments had a significant positive association with CHCl₃-extracted chemical classes, i.e. unsaturated hydrocarbons and lipids, with the coupled application (stover and cover crop), and lignin and proteins with the corn stover only treatment. Taken together, our study shows how different C addition practices influence the molecular composition of SOM and the structure of soil microbial communities.

1. Introduction

Corn (*Zea mays* L.) residue has served as a feedstock for cellulosic ethanol production in the United States because of its high cellulose content and easy availability (Blanco-Canqui and Lal, 2009; Stewart et al., 2018), but this results in removal of corn stover and hence reduced carbon (C) sequestration in soil. Studies have suggested that the threshold levels of residue removal must be assessed for principal soil types based on the needs to maintain or enhance soil productivity. Besides, agronomic strategies such as cover crops, diverse crop rotations, and manure application, are employed to compensate the C loss by

stover removal.

Planting a cover crop between periods of regular crop production increases soil organic matter (SOM) by adding biomass C and improving soil aggregation to protect SOM (Ruis and Blanco-Canqui, 2017; Stetson et al., 2012). In north central USA, winter rye (*Secale cereale*) is suggested as a preferred cover crop species because it is cold tolerant, vigorous, and cost-effective (Martinez-Feria et al., 2016). In addition, winter rye meshes well with a corn crop in the winter niche (Wilke and Snapp, 2008). C inputs from rye could replace some of the C removed in stover, although its capacity to directly increase SOM varies with agricultural management and soil type (Austin et al., 2017; Villamil et al.,

* Corresponding author.

E-mail address: tiedje@msu.edu (J.M. Tiedje).

¹ Present address: School of the Environment, Washington State University, Richland, WA, 99354, USA.

² Present address: Department of Environmental Science, University of Arizona, Tucson, AZ, 85721, USA.

2006; Cates and Jackson, 2019). Given the advantages of the rye cover crop with corn and its potential substitution for stover, we sought to evaluate these treatments at a more detailed biochemical and microbiological level.

SOM responds to organic amendments by shifting its quality and quantity, but defining its molecular composition and functional properties is still a challenge, in part because of the vast array of compounds. SOM exists on a humification continuum; operationally defined extractions have been extensively used to fractionate SOM into different classes to reduce heterogeneity (Ohno et al., 2010). Diverse technologies have been applied to elucidate the structure and function of the derived fractions after extraction, from chemical compositions of SOM (i.e. humin, humic acid, and fulvic acid) (Stevenson, 1994), to the fluorescence spectroscopy based on the multiple peaks and their specific location (Sanchez et al., 2013). However, the molecular information of the components of SOM fractions are still lacking in most studies (Kim et al., 2003). In the past decade, electrospray ionization (ESI) coupled with Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) has become a useful method because it offers unparalleled mass resolving power (>1 M) and mass measurement accuracy (<1 ppm). The organic compounds can be assigned to thousands of molecular formulas of known natural organic matter (Tfaily et al., 2015). In this way, it is possible to identify the chemical compositions of extremely complicated samples (Choi et al., 2017; Guigue et al., 2016; Wu et al., 2018). FT-ICR-MS has to date been proven feasible in distinguishing organic compounds in both terrestrial and aquatic environments, such as paddy soil and sediments as well as groundwater (Ajaero et al., 2017; Herzprung et al., 2016; Li et al., 2018; Tfaily et al., 2015; Zhang et al., 2016).

The process of SOM decomposition should share some relationship to the microbial community that catalyzes its formation, e.g. the microbial taxa that respond differently to the different incorporated organic materials (Zheng et al., 2018). Generally, easily degradable C compounds have a higher proportion of common microbial taxa responsible for its mineralization (Yan et al., 2018). In this regard, we hypothesized that different agronomic strategies, such as corn stover retention and cover crop, because of their very different C constituents at the time of C return to soil, would result in differences in soil bacterial community structure as well as in the enrichment of chemical classes of extractable SOM. To test this, we sampled a field experiment with treatments of corn stover retention, rye cover crop, both, and neither at the W.K. Kellogg Biological Station (Michigan, USA). The objectives were to unravel: 1) the extent to which the corn stover and cover crop influence SOM and the identities of its extractable components; 2) the responsiveness of bacterial and fungal communities to the four treatments and 3) whether there are SOM compounds indicative of different agronomic practices. This knowledge will improve our understanding of the factors regulating the soil microbial community in a biofuel or similarly managed agricultural ecosystem.

2. Material & methods

2.1. Field sites and sample overview

The experiment site is located at the Great Lakes Bioenergy Research Center (GLBRC) Biofuels Cropping System Experiment (BCSE) at Kellogg Biological Station in southwest Michigan, USA. The BCSE experimental design utilizes a randomized complete block design with 5 replicate blocks (30 × 40 m). The site had the same or very similar cropping history prior to establishing the current plot design in 2008. The soil is predominantly a Kalamazoo loam (Fine-Loamy, Mixed, Semiactive, Mesic Type Hapludalf), with 47–56% sand (Austin et al., 2017). There are a total of ten biofuel cropping systems as described previously (Zhang et al., 2017) (referred to <https://lter.kbs.msu.edu/maps/images/20170316-glbrc-kbs-bcse-map.pdf>), of which two corn systems were selected for current study: continuous corn (*Zea mays*) (G1) and

continuous corn + rye cover crop (*Secale cereal*) (G2). For each replicate in G1 system, the corn stover in main plot was removed after harvest (control), and in the subplot the corn stover was left in the field (denoted as CS). For each replicate in the rye cover crop G2 system, the corn stover was removed in the main plot (denoted as CC), and all of the stover was left in the subplot (denoted as CSCC). The cover crop was harvested just prior to corn planting in the spring and its aboveground biomass removed as part of the treatment's biofuel yield. The subplots were established for the 2012 season so had experienced 5 years treatment different from the main plots which had experienced 9 years of their treatments.

Soil samples were taken February 20, 2017, during a winter soil thaw. Soil temperature was below 4 °C limiting current microbial growth. Five randomly distributed cores (2-cm diameter) of 10 cm depth were composited to form a replicate in each plot/subplots, and there were five replicates collected for each treatment. Soil samples were then put into a container of dry-ice immediately and transported to the laboratory within 3 h after collection. In the laboratory, samples were divided into two portions: one portion was air-dried for determination of total SOM content. The other was frozen at −80 °C for later SOM chemical composition characterization, phospholipid fatty acid (PLFA) determination, and DNA extraction.

On November 22, 2017, a month after the harvest/retention of corn stover and the planting of rye, we collected a second set of soil samples from the same subplots/plots following the identical procedure for a repeated determination of total SOM content. This timing was chosen to represent the un-frozen stage of soil and to minimize the influence of the fresh C input.

2.2. Soil total organic matter

The air-dried soil samples were sent to Michigan State University Soil and Plant Nutrient Laboratory to determine the SOM content using the K₂CrO₄ external heating method (<http://extension.missouri.edu/explorepdf/specialb/sb1001.pdf>).

2.3. Solvent extraction for SOM

A portion of the frozen soil samples were sent to Environmental Molecular Sciences Laboratory (EMSL) at Pacific Northwest National Laboratory (Richland, WA, USA) for molecular determination of SOM components.

Previously, different sequential extraction protocols have been compared to reveal SOM fractions by Tfaily et al. (2017). Afterwards, an optimized sequential protocol, i.e. water, methanol (MeOH), and chloroform (CHCl₃), is adopted by Graham et al. (2017) for river sediments. In this study, the identical sequential extraction protocol was employed for agricultural soil samples. According to Tfaily et al. (2015, 2017), each solvent is selective toward specific types of compounds. Water is the natural *in situ* extractant with a selection bias for carbohydrates with high O/C ratios, amino sugars, and other labile polar compounds. The water-extracted organic matter represents the most labile fraction of SOM and approximates the dissolved organic matter found in soil solution (Ohno et al., 2010). CHCl₃ is selective for nonpolar lipids associated with mineral interactions and cellular membranes (i.e., physically bound OC). MeOH has a polarity in between that of water and CHCl₃, and it thus extracts both water-soluble and bound-OC pools. The compositional overlap exists between water-soluble and MeOH extracted OC pools (Tfaily et al., 2015; Graham et al., 2017). The samples were prepared by adding 1 mL of solvent to 1–100 mg bulk soil and shaking in 2 mL capped glass vials for 2 h on an Eppendorf Thermomixer. Samples were removed from the shaker and left to stand before spinning down and pulling off the supernatant to stop the extraction. The soil residue was dried with nitrogen gas to remove any residual solvent, and then solvent MeOH and solvent CHCl₃ were added sequentially.

2.4. FT-ICR-MS data acquisition and data analysis

Molecular compositions of SOM in the soil extracts were determined by a 12 T FT-ICR-MS (Bruker Daltonics Inc., Billerica, MA, USA), a DOE-BER national user facility located at EMSL.

The detailed process has been described by Tfaily et al. (2015) and Graham et al. (2017). Briefly, samples were introduced to the electrospray ionization source equipped with a fused silica tube through a syringe pump with the optimal parameters established in earlier dissolved organic matter (DOM) characterization experiments (Tfaily et al., 2015). All sample peak lists for the entire dataset were aligned to each other prior to formula assignment to facilitate consistent peak assignments and eliminate possible mass shifts that would impact formula assignment. Putative chemical formulas were assigned using the Compound Identification Algorithm (CIA) described by Kujawinski et al. (2006). Chemical formulas were assigned based on the following criteria: S/N > 7, and mass measurement error < 1 ppm, taking into consideration the presence of C, H, O, N, S and P and excluding other elements (Tfaily et al., 2017).

FT-ICR-MS *m/z* intensities were converted into presence/absence data prior to analysis because differences in *m/z* intensity are influenced by ionization efficiency as well as relative abundance (Graham et al., 2017). This approach avoids biases incurred by different ionization efficiencies for different types of compounds and potential interferences between compounds or from complexation with metals (Boye et al., 2017). Then, from the formula assignment, the number of peaks of each class, i.e. the relative abundance, was calculated and used for the downstream analysis. Note that the relative abundance was used here as that is the convention used by chemists for this type of data but in the biological sense it can be viewed as a richness, i.e., the number of compounds.

To interpret the large data set, van Krevelen diagram is often plotted on the basis of the molar H/C ratios (*y* axis) and molar O/C ratios of the assigned compounds (Kim et al., 2003). It provide a means to compare the average properties of SOM and enable identification of the major biochemical classes. Compounds with similar structural characteristics fall within the same region within a van Krevelen plot (Zhang et al., 2016). For this study, the chemical compounds were grouped into the eight main families: lipids ($0 < \text{O/C} \leq 0.3$, $1.5 \leq \text{H/C} \leq 2.5$), proteins ($0.3 < \text{O/C} \leq 0.55$, $1.5 \leq \text{H/C} \leq 2.3$), amino sugars ($0.55 < \text{O/C} \leq 0.7$, $1.5 \leq \text{H/C} \leq 2.2$), carbohydrates ($0.7 < \text{O/C} \leq 1.5$, $1.5 \leq \text{H/C} \leq 2.5$), unsaturated hydrocarbons ($0 \leq \text{O/C} \leq 0.125$, $0.8 \leq \text{H/C} \leq 1.5$), lignin ($0.125 < \text{O/C} \leq 0.65$, $0.8 \leq \text{H/C} < 1.5$), tannins ($0.65 < \text{O/C} \leq 1.1$, $0.8 \leq \text{H/C} < 1.5$), and condensed hydrocarbons ($0 \leq \text{O/C} \leq 0.95$, $0.2 \leq \text{H/C} \leq 0.8$).

In this study, pairwise comparison was carried out between two contrasting treatments in order to show the effect on SOM compositions of corn stover (i.e. control vs CS, and CC vs CSCC) and cover crop (control vs CC, and CS vs CSCC). For each paired group, one treatment was referred to the “counterpart” of the other. We defined the “unique” SOM compounds based on the following criterion: the chemical formulas that appeared in all the five replicates of a treatment but none of the other. Then, the “unique” SOM compounds were visualized on van Krevelen diagrams. In this way, we could highlight the types of compounds that enriched or disappeared between the paired treatments.

Aromaticity index (AI) is an indication of the refractory behavior of molecules against biodegradation (Guigue et al., 2016). It considers the possibility that heteroatoms (in particular O) can form double bonds not contributing to the aromaticity (Koch and Dittmar, 2006). Less aromatic compounds reflect potentially higher biodegradability of SOM (Choi et al., 2017). In this study, AI was employed to interpret the presence of aromatic structures in a molecule after pooling all the chemical formulas of SOM from three solvents together. We calculated the AI from the number of atoms according to Eqn. (1):

$$\text{AI} = (1 + \text{C} - \text{O} - 0.5\text{H}) / (\text{C} - \text{O} - \text{S} - \text{N} - \text{P}) \quad (1)$$

Where C, O, S, H, N, and P represented the number of C, O, S, H, N, P atoms of each compound, respectively (Koch and Dittmar, 2006). The compounds with values of AI > 0.5 are expected to be aromatic species (Choi et al., 2017).

2.5. Phospholipid fatty acid (PLFA) analysis

A portion of freeze-dried soil samples were analyzed for PLFAs by Microbial ID, Inc. (MIDI, Newark, DE, USA). Methods for extraction were adapted from company's standard procedure.

Individual fatty acid methyl esters (FAMES) were identified and quantified using the MIDI Sherlock Microbial Identification System (MIDI, Newark, Delaware, USA). The result for each individual fatty acid was expressed as a percentage of the total amount of fatty acids (mol%) found in a given sample. The combined masses of FAMES reported as typical of fungi (18:2 ω 6c), general bacteria (15:0; i15:0; a15:0; i16:0; 16:0 ω 9; i17:0; a17:0; cy17:0; 18:1 ω 7; cy19:0), Gram-negative bacteria (14:0; 16:1 ω 6c; 17:1 ω 8c; 18:1 ω 7c; cy17:0; cy19:0; 20:1 ω 9c; 21:1 ω 8c; 21:1 ω 3c; 22:1 ω 3c), Gram-positive bacteria (15:1 ω 9c; 15:1 ω 6c; 15:1 ω 9c; i15:0; a15:0; 16:0; 17:1 ω 9c; a17:0; 18:0; 17:1 ω 9c), Actinobacteria (10Me16:0; 17:1 ω 7c; 10Me17:0; 18:1 ω 7c; 10Me18:0), arbuscular mycorrhizal fungi (AMF; 16:1 ω 5c) were used as signatures for these microbial groups. Fungal and bacterial markers were used to calculate fungal to bacterial biomass ratio (F:B).

2.6. DNA extraction, PCR amplification, and sequencing

Soil microbial DNA was extracted from the frozen soil samples using PowerMax Soil DNA Isolation Kit (MO BIO, CA, USA) following the manufacturer's protocol and quantified using the Qubit dsDNA HS Assay Kit (Life Technologies, OR, USA) with a Qubit fluorometer (Invitrogen, OR, USA). The extracted DNA was used for quantitative analysis and for 16S rRNA gene amplicon sequencing.

2.7. 16S rRNA gene amplicon sequencing and data analysis

The V4 region of the 16S rRNA genes was sequenced with a one-step PCR library preparation strategy. Briefly, the PCR was carried out with the target-only primer pair 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') (Caporaso et al., 2011). In the second-round PCR, phasing primers with Illumina functionalities, spacers, as well as barcodes on the reverse primers were introduced. Sample libraries were generated from purified PCR products and pooled for sequencing as reported previously (Zhang et al., 2017).

To control variation resulting from an unequal number of sequences across samples, sequence resampling was performed for each sample after OTU generation at a rarefaction sequence level based on the sample with the fewest number of sequences. Sequences from each sample were randomly drawn from the original pool until the rarefaction sequence level is achieved. Once a sequence is drawn, it is excluded from further rounds of selection to prevent repetition.

After sequencing was completed, 16S rRNA gene data were processed using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline using default parameters unless otherwise noted (Caporaso et al., 2010). Reads below a quality score of 25 and 200 bp in length were trimmed, and then assigned to soil samples based on unique 5-bp barcodes. Operational Taxonomic Units (OTUs) were selected using the UPARSE pipeline with a sequence similarity cut-off of 97% (USEARCH software V8) (Edgar, 2017); chimeric sequences were also removed. Taxonomy was assigned to OTUs against a subset of the Silva 119 database (<http://www.arb-silva.de/download/archive/qiime/>) using PyNAST. The 16S rRNA gene sequencing data have been deposited at DNA Data Bank of Japan (DDBJ) under accession number DRA010263.

2.8. Statistical analysis

One-way analysis of variance (ANOVA) followed by Tukey's HSD test was used to determine the effects of corn stover and cover crop on SOM content, 16S rRNA gene copy number, PLFAs, and the relative abundances of bacterial community compositions among different treatments. Significant differences were accepted at $P < 0.05$.

For the changes in soil bacterial community and SOM structure, the Bray-Curtis distances were computed based on the OTU tables and the total amount of SOM biochemical classes, and were then visualized with non-metric multidimensional scaling (NMDS) plots using the "metaMDS" function (vegan package) in R (Version 3.6.0). Permutational multivariate analysis of variance (PERMANOVA) was conducted to detect pairwise differences in group distances by using "adonis" function (vegan package, 999 permutations). The relative importance of the SOM compounds dissimilarities contributing to the variation in the soil bacterial taxonomic compositions (OTU level) was further identified by redundancy analysis (RDA) and a subsequent Monte Carlo test (999 permutations), which was performed using "rda" and "permutest" function of vegan package (Bray-Curtis distances). The goodness-of-fit (R^2) and associated statistical significance (P-value) of each SOM compound were verified using "envfit" function in vegan.

3. Results

3.1. SOM content

The total SOM contents varied from 1.6% to 3.4%, with the average values listed in Table 1. The highest value was found in CSCC and the lowest in the control, although the P-value for comparisons among treatments just exceeded usual statistical thresholds for significance (ANOVA, $P = 0.051$). Similar trends were observed for the November samples in the same year of 2017.

3.2. Differences in number of components extracted by the three solvents

The richness of compounds (peak number) varied with the extracting solvents and organic amendments as well. Generally, CHCl_3 extracted more SOM compounds than MeOH and water (Fig. 1). In the water extracts, the control had the most peaks (1,110), followed by CS (915) and CC (897). The lowest number of peaks was observed in CSCC (637), significantly different from the control as well as CS and CC ($P = 0.001$). It was further found that the relative abundances of chemical classes decreased in the order: condensed hydrocarbons > lignin > lipids > proteins (Fig. S1). In the MeOH extracts, no significant differences were observed among treatments ($P = 0.215$, Fig. 1), and the most abundant class was lipid, followed by protein and lignin (Fig. S1). For the CHCl_3 extracts, only CSCC (2,250) had significantly fewer peaks in comparison with the control (2,920, $P = 0.01$). Similar to the distribution in the MeOH extracts, lipid was the most abundant (Fig. S1).

Table 1

Total SOM contents in different amendments. Control, corn stover is removed after corn harvested. CS, corn stover retention. CC, both corn stover and cover crop are removed after corn harvested. CSCC, cover crop is removed but corn stover remains after corn harvested. Different letters within each column denote significant differences at $P < 0.05$.

Treatment	SOM content (%)	
	February sample	November sample
control	2.12 ± 0.33 a	1.84 ± 0.29 a
CS	2.32 ± 0.60 a	2.10 ± 0.21 a
CC	2.48 ± 0.37 a	2.52 ± 0.13 a
CSCC	2.64 ± 0.54 a	2.60 ± 0.43 a

3.3. Aromaticity compounds

The percentages of aromatic compounds ($\text{AI} > 0.5$) varied substantially in different extracts, with the average highest in water, followed by MeOH and CHCl_3 (Fig. 2), in line with each solvent's extractive traits. Different agronomic practices produced differences in the proportions of $\text{AI} > 0.5$ compounds. CS significantly decreased the proportion of $\text{AI} > 0.5$ compounds compared to the control ($P < 0.05$) in water and MeOH. When cover crop present, there was no significant difference due to stover retention, CC vs CSCC. By contrast, the cover crop's presence with water extracts showed an increasing proportion of $\text{AI} > 0.5$ when compare to the control, as well as with stover, CSCC vs CS ($P < 0.05$).

3.4. Unique SOM compounds

Van Krevelen plots, together with Venn diagrams, were employed to show the SOM compound characteristics (Fig. 3 and Fig. S3). Among all treatments, the common compounds only accounted for 19.8%, 18.4%, and 0.1% in the water, MeOH, and CHCl_3 extracts, respectively (Fig. S2). The unique compounds varied with the solvents and the organic amendments. Specifically, in the water extracts, the unique compounds in the control accounted for 17.4% of total compounds, larger than other treatments, and those in CSCC accounted for the least (0.4%). In the MeOH extracts, large proportions of unique compounds were observed in CS and CSCC (28.7% and 29.4%, respectively), while those in the control and CC accounted for only 3.8% and 0.6%, respectively. In the CHCl_3 extracts, the proportions of unique compounds ranged from 3.9% (control) to 25.6% (CS).

Pairwise comparison between the contrasting treatments showed that the unique SOM compounds varied depending on treatments and extracting solvents, as illustrated by van Krevelen diagrams and the derived stacked plots (Fig. 3 and Fig. S4). Specifically, in the water extracts (Fig. 3-a), the augmented treatment (CS or CC) generated fewer compounds in comparison with the control, which contained a broad range of compounds, including protein-, lignin-, and carbohydrates-like compounds. CS appeared to have some unique lignin-like compounds, while CC led to the enrichment of condensed hydrocarbons.

In the MeOH extracts, it was notable that no unique compound appeared in CS or CC as compared to the control that harbored various unique ones (Fig. 3-b1 and 3-b3). By contrast, CSCC produced more kinds of unique compounds, including lipids, proteins, and carbohydrates, in comparison with either CS or CC (Fig. 3-b2 and 3-b4), suggesting that CSCC had an enhancing effect.

In the CHCl_3 extracts, the most abundant unique compounds were lipid- and protein-like, in agreement with the solvent trait (Fig. 3-c). However, the pattern of unique compounds produced by corn stover diverged from that by cover crop. For example, CS harbored more unique compounds than the control (Fig. 3-c1), a similar trend observed between CSCC and CC (Fig. 3-c2). By contrast, CC possessed less unique compounds than the control (Fig. 3-c3), as well as CSCC compared to CS (Fig. 3-c4).

NMDS analysis based on the chemical classes of SOM from three solvents showed that only CSCC separated from other treatments (Fig. 4, PERMANOVA, $P = 0.045$), reflecting the strongest effect of coupled application of stover retention and cover crop.

3.5. Microbial and functional group lipid biomass

PLFA analysis results showed that most of the microbial and functional group lipid biomass were generally similar among treatments, including total biomass, eukaryote, fungi, and bacteria (Table 2). Lipid marker for arbuscular mycorrhizal fungi (AMF) was significantly higher in CSCC (4.53%) and CC (4.57%) than in the control (4.12%). Considering that the soil was dominated by bacteria rather than fungi (F/B ratio was 0.065–0.072), we focused on the bacterial community in the follow-up analysis.

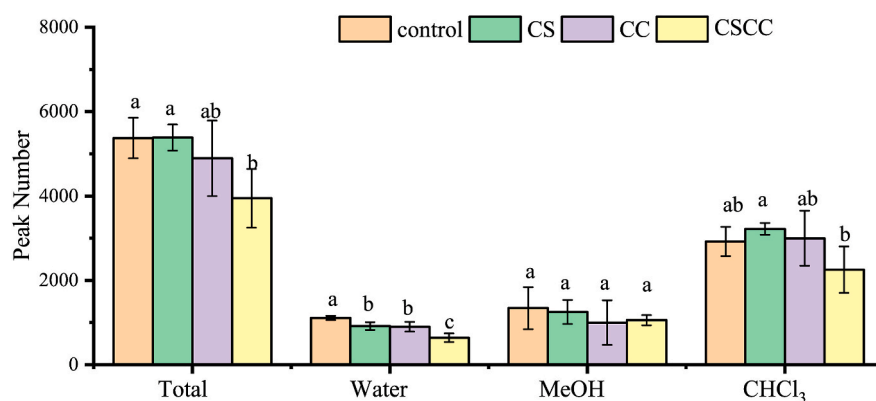


Fig. 1. Peak numbers in water, MeOH, and CHCl₃ extracts determined by ESI-FT-ICR-MS. Letters denote significant differences within each solvent according to ANOVA with Tukey's post-hoc test. Control, corn stover is removed after corn harvested. CS, corn stover retention. CC, both corn stover and cover crop are removed after corn harvested. CSCC, cover crop is removed but corn stover remains after corn harvested.

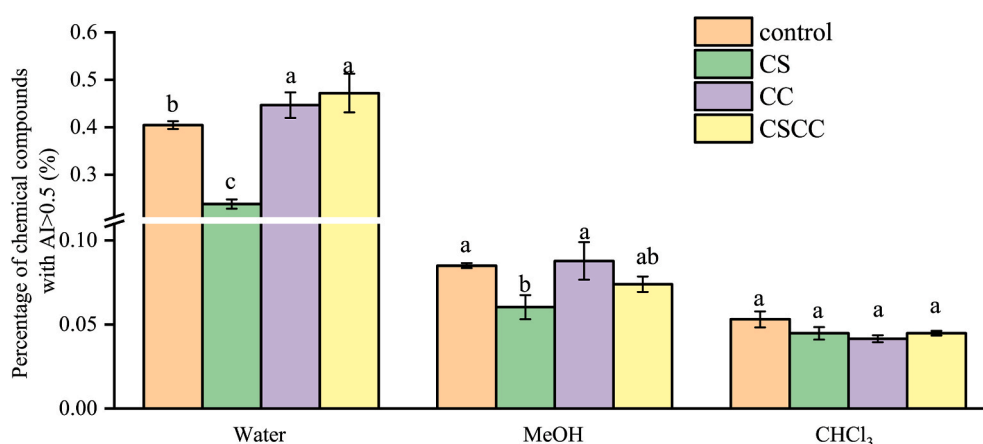


Fig. 2. Percentages of SOM biochemical classes with AI > 0.5 in water, MeOH, and CHCl₃ extracts analyzed by FT-ICR-MS. Letters denote significant differences within each solvent according to ANOVA with Tukey's post-hoc test.

3.6. Bacterial population in soil

Real-time qPCR results showed that the bacterial population in soil ranged from 2.1×10^9 to 8.95×10^9 gene copies per gram (Table 2), although no significant differences were found among all treatments according to the one-way ANOVA (Tukey's test, $P = 0.526$).

3.7. Soil bacterial community composition

Taxonomic analysis of 16S rRNA gene amplicons showed that Proteobacteria was the most abundant phylum (23.2–34.1%), followed by Acidobacteria (10.56–25.8%), Actinobacteria (3.55–11.3%), Verrucomicrobia (4.97–12.39%), Bacteroidetes (3.67–13.29%), Planctomycetes (2.15–7.09%), Gemmatimonadetes (1.15–2.88%), and Firmicutes (0.43–3.26%). Several phyla were shifted by the amendments in term of their relative abundances (Fig. 5). For example, a significantly lower proportions of Acidobacteria ($P = 0.001$), Nitrospirae ($P = 0.014$), and Armatimonadetes ($P = 0.008$) were found in CSCC, compared to the control. Further, CS had a significantly lower abundance of Actinobacteria than the control ($P = 0.045$).

At the finer levels, some lineages had distinct responses to different field management (Table 3). In particular, CSCC decreased significantly the occurrences of Acidobacteria GP6 ($P = 0.041$), Clostridia ($P = 0.001$), and Nitrospira ($P = 0.009$), but significantly increased those of Sphingobacteria ($P = 0.043$) and Thermomicrobia ($P = 0.009$) in comparison with the control. By contrast, CS increased significantly the proportion of Myxococcales ($P = 0.021$), but significantly decreased that

of Clostridia ($P = 0.041$) as compared to the control.

Non-metric multidimensional scaling (NMDS) ordination at the OTU level (Bray-Curtis dissimilarity matrices) demonstrated the discrepancy of the bacterial communities among treatments (Fig. S4). Especially, distinct separations were found between control vs CS ($R^2 = 0.22$, $P = 0.03$), control vs CSCC ($R^2 = 0.32$, $P = 0.03$), and CS vs CSCC ($R^2 = 0.32$, $P = 0.042$).

3.8. Relationship between soil bacterial community and SOM compounds

The RDA biplot (Fig. 6) showed that the SOM compounds in all the extracts explained 38.5% of variability in the bacterial community composition. The canonical coefficients, the goodness-of-fit (r^2) and associated statistical significance (P -value) of each SOM compound is in Table S1. Along the axis 1, some CHCl₃-extracted unsaturated hydrocarbons ($r^2 = 0.63$, $P = 0.001$) and lipids ($r^2 = 0.37$, $P = 0.020$), were significantly correlated with CSCC, while some CHCl₃-extracted lignin ($r^2 = 0.41$, $P = 0.014$) and proteins ($r^2 = 0.38$, $P = 0.019$) had significantly positive relationship with CS.

4. Discussion

In this study, the amendments of stover retention and cover crop, singly and in combination, did not lead to significant changes in total SOM content after 5–9 years but positive trends were apparent, especially versus the control. This sluggish response is in accordance with some previous studies. Examples include a 13-year addition of a winter

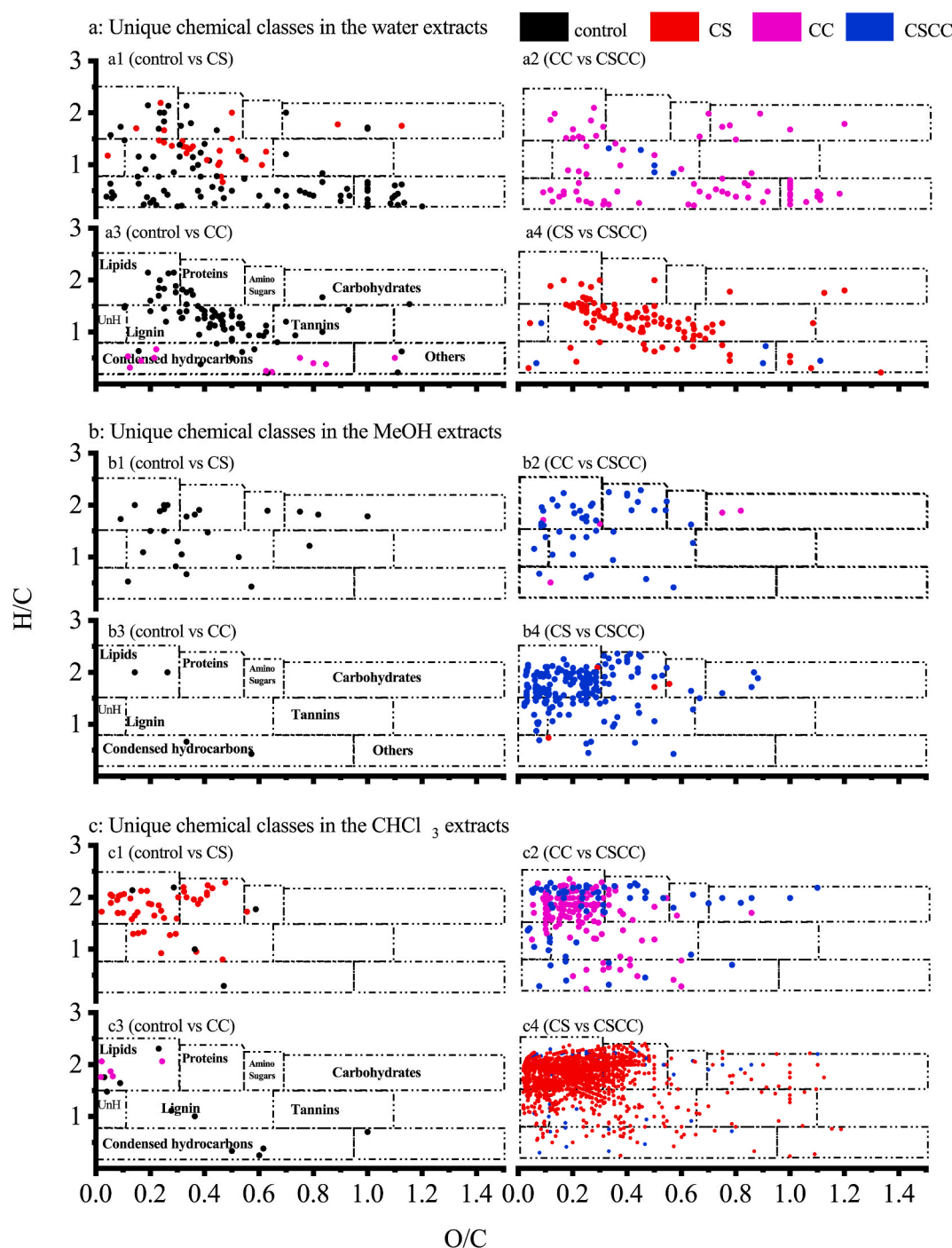


Fig. 3. Pairwise comparisons showing the unique compounds of SOM in two contrasting treatments extracted by water (a), MeOH (b), and CHCl_3 (c) using van Krevelen diagrams. Colors indicate in which treatment that compound is unique to the other. Subplots of a1, b1, c1 indicate the pairwise comparison between CS and control. Subplots of a2, b2, c2 indicate the pairwise comparison between CSCC and CC. Subplots of a3, b3, c3 indicate the pairwise comparison between CC and control. Subplots of a4, b4, c4 indicate the pairwise comparison between CSCC and CS. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

cereal rye cover crop at three locations in Maryland, USA (Steele et al., 2012), and the study of retaining residue in the short term (<10 years) (Blanco-Canqui and Lal, 2009). However, there are also some findings showing gains in SOM contents after the organic amendments (Urra et al., 2018; Warren Raffa et al., 2015; Wegner et al., 2018). We assumed such divergences might derive from the edaphic properties, climates, amending strategy, and management history.

4.1. Organic amendments decreased the richness of SOM compounds and changed the occurrence of unique compounds

Increasing organic matter inputs generally increases soil organic matter content (West and Six, 2007). If organic matter inputs increase microbial activity, we expect that the increased microbial biomass and necromass will contribute to soil organic matter formation (Six et al., 2006), with some of it contributing to the stable mineral fraction (Cotrufo et al., 2015). In this sense, we hypothesized that an increase in

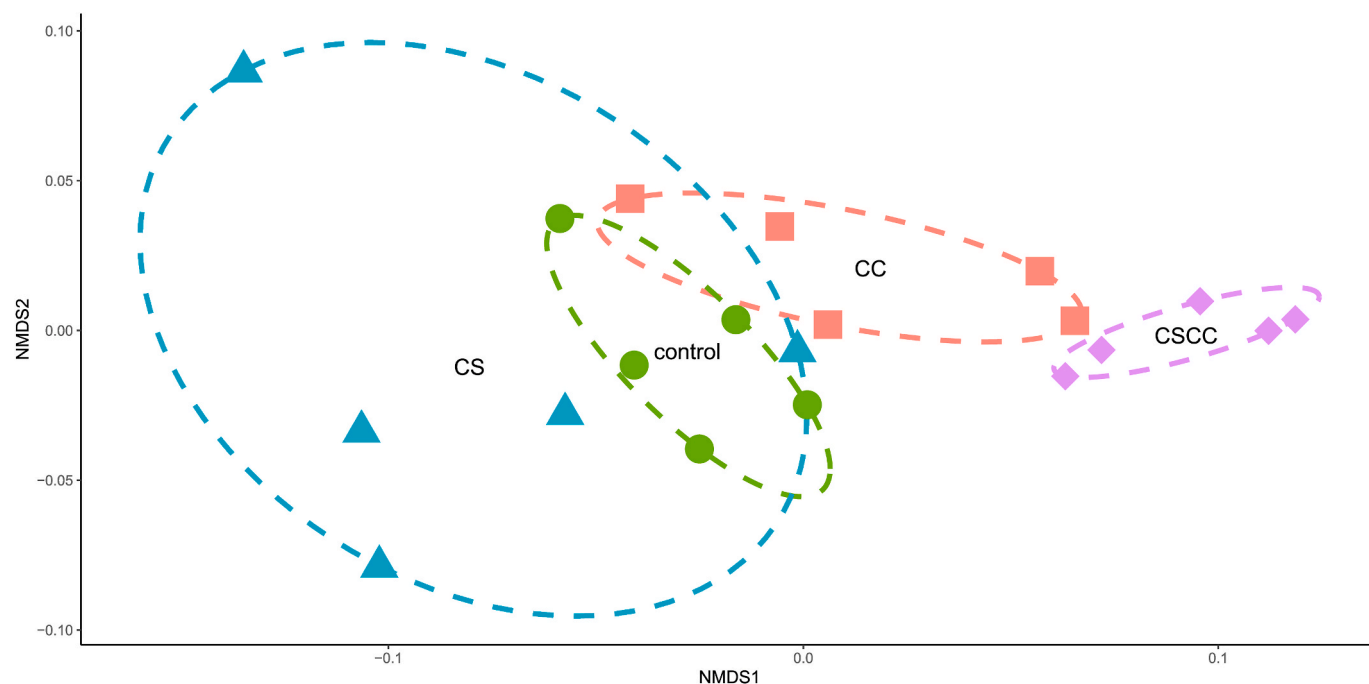


Fig. 4. NMDS plot from the SOM chemical classes detected by FT-ICR-MS.

Table 2

The proportions of lipid groups in different treatments as determined by lipid analysis and 16S rRNA gene copy number determined by qPCR. Control, corn stover is removed after corn harvested. CS, corn stover retention. CC, both corn stover and cover crop are removed after corn harvested. CSCC, cover crop is removed but corn stover remains after corn harvested. Different letters within each column denote significant differences at $P < 0.05$.

	TB (nmol/g)	AMF %	Eukaryote %	Fungi%	Actinobacteria %	F/B	Fungi	Bacteria	gene copies*10 ⁹ /g
control	69.57 a	4.12 b	1.32 a	1.06 a	18.08 a	0.070 a	6.51 a	93.49 a	5.92 a
CS	64.60 a	4.22 ab	1.62 a	0.73 a	17.24 a	0.065 a	5.78 a	93.98 a	3.67 a
CC	69.68 a	4.57 a	1.36 a	0.92 a	17.72 a	0.072 a	5.76 a	92.89 a	4.30 a
CSCC	64.41 a	4.53 a	1.38 a	0.87 a	17.58 a	0.064 a	5.31 a	93.87 a	4.15 a

TB, Total Biomass; AMF, Arbuscular Mycorrhiza Fungi; F/B, Fungi/Bacteria.

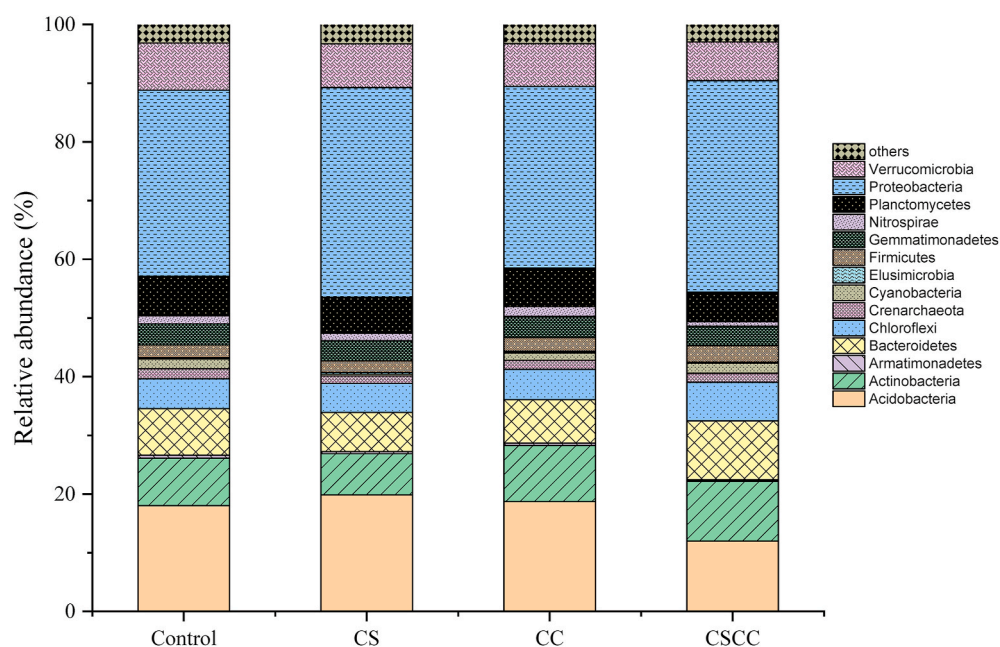


Fig. 5. The stack column chart of the relative abundances of dominant bacterial phyla derived from 16S rRNA gene sequencing.

Table 3

The relative abundances of the different lineages in soil bacterial community. Control, corn stover is removed after corn harvested. CS, corn stover retention. CC, both corn stover and cover crop are removed after corn harvested. CSCC, cover crop is removed but corn stover remains after corn harvested. Different letters within each column denote significant differences at $P < 0.05$.

	Actinobacteria (%)	Acidobacteria Gp6 (%)	Clostridia (%)	Nitrospirales (%)	Thermomicrobia (%)	Sphingobacteriia (%)	Burkholderiales (%)	Myxococcales (%)
control	13.15 ab	7.95a	0.21a	0.56 ab	0.02b	3.50b	2.00b	2.83b
CS	10.47b	7.16a	0.06b	0.65a	0.01b	4.03b	1.62b	4.20a
CC	14.82 ab	8.32 a	0.09b	0.72a	0.05b	3.56 b	2.19b	3.05 ab
CSCC	16.52a	4.19b	0.11 b	0.32b	0.11a	7.48 a	3.67a	2.93 ab

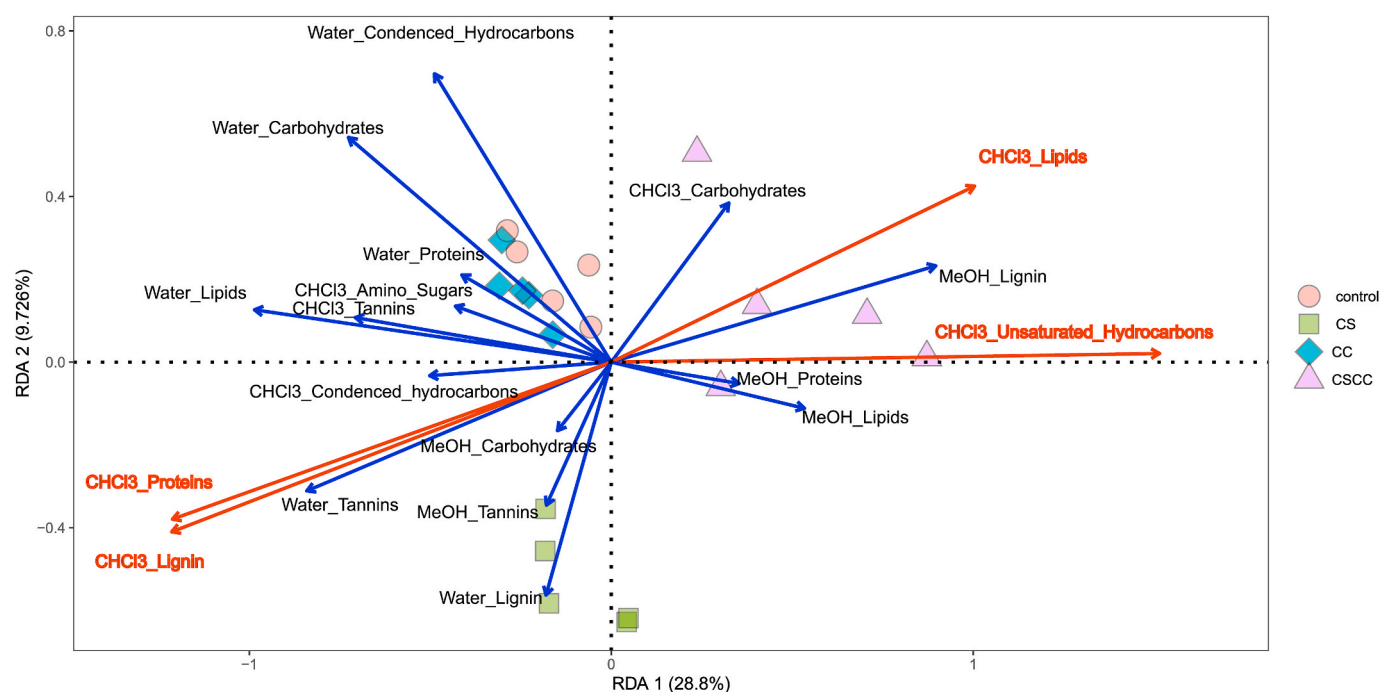


Fig. 6. Multivariate analysis of soil microbial community and SOM compounds using redundancy analysis (RDA). Ordinations are based on Bray-Curtis, which utilizes relative OTU abundance information of soil bacterial community. SOM biochemical classes are fit to the ordinations.

SOM would be accompanied by more unique SOM compounds. Surprisingly, contrary results were found especially in water and MeOH extracts (Figs. 2 and 3). This outcome is presumably the result of microbial activity and community selection over the decade of treatments. We offer two explanations, perhaps related. First, the fresh organic treatments would provide a priming effect, thereby stimulating the degradation of current and some of the old C compounds leaving less to be extracted (Blagodatskaya and Kuzyakov, 2008; Pegoraro et al., 2019). Second, the 5-9-year selection of copiotrophs on the new C produced metabolites common in soil (i.e. less unique SOM compounds). By contrast, the old, recalcitrant organic compounds in non-amended soil served as the main C and energy sources for microorganisms (especially oligotrophs), resulting in diverse metabolites or more persistent residues (i.e. more unique SOM compounds). This is supported by a previous study that confirms the depletion of recalcitrant SOM fractions in stover-removed soil based on quantitative ^{13}C NMR (Stetson et al., 2012), a sign of the role of recalcitrant SOM compounds for microorganisms. Also, the increased proportion of AI>0.5 compounds in the control of this study (Fig. 2) is consistent with such a process, because the degradation of aromatic ring cleavage would lead to an increase of aromatic C and/or condensed aromatic structures (Derrien et al., 2017).

4.2. Stover retention and cover crop influenced SOM compounds differently

For a given site with similar soil type, the initial quality of organic materials is intimately related to their decomposition process. Generally, corn stover has a higher C/N ratio (e.g. 79.5 reported by Yang et al. (2017)) than rye cover crop (e.g. 22.2 by Barel et al. (2018)). Such differences will lead to the variance in aromaticity (Fig. 2) and the unique SOM compounds, as revealed by pairwise comparison in this study (Fig. 3).

Stover, representing the dead plant residue, led to fewer aromatic compounds (Fig. 2). This phenomenon implied that the less humified pools of soil C are not being replenished in the stover retention treatment (Stetson et al., 2012). Similar trends are observed in other studies (Chen et al., 2017; Song et al., 2017). In addition, according to the van Krevelen plots, the unique compounds generated by stover retention were primarily lignin-like (in the water and CHCl_3 extracts) and lipid-like (in the CHCl_3 extracts) (Fig. 3). Lignin-like compounds are likely derived from allochthonous terrestrial plant sources, and represent the bulk of the semi-labile and refractory SOM pool (Lusk et al., 2016). By contrast, lipid-like compounds are aliphatic and microbially derived, and typically represent the bulk of the labile pool (Hendrickson et al., 2007). Two different pathways/stages of SOM formation are proposed by Cotrufo et al. (2015) after litter incorporation. At the early stage, the non-structural compounds are lost from the litter, and the acid

un-hydrolysable fraction (generally defined as 'lignin') increases in absolute amounts (though different from the types of SOM in this study), owing to the incorporation of microbial residues in this fraction. At the late stage, physical transfer of the residue to the mineral soil takes effect, resulting in no preferential loss of any chemical compounds and no incorporation of microbially produced residue C. In this respect, we suggest that early stage SOM formation is occurring in this study ~3 months after stover retention. At this stage, microbial decomposition produces necromass which includes refractory structures like lipids and mineral bound lipids.

On the contrary, cover crop tended to influence SOM compounds in a different way, i.e., the increased proportion of $AI > 0.5$ compounds and the emergence of unique condensed hydrocarbons (Figs. 2 and 3). We presume that this is owing to the involvement of plant activity, i.e. the rhizosphere effect. In rhizosphere samples, compounds with aromatic structures and less aliphatic are more abundant than in non-rhizosphere samples, resulting in a more recalcitrant C pool (Wen et al., 2018). A previous study also shows the increased proportion of $AI > 0.5$ compounds by rye cover crop based on diffuse reflectance Fourier transform infrared (DRIFT) spectra (Ding et al., 2006), which was in good agreement with current study, particularly in the water extracts (Fig. 2). Further illustration of unique SOM compounds (Fig. 3) unraveled that cover crop garnered more condensed hydrocarbons (water extracts) and lipids ($CHCl_3$ extracts), which, we assume, were also intimately associated with the plant, especially root exudates.

4.3. Microorganisms' response to stover retention and cover crop

Neither stover retention nor cover crops changed the total microbial biomass, bacterial biomass or 16S rRNA gene copies as revealed by PLFA and qPCR. However, some functional microbial groups were altered. For example, the lipid biomarker of AMF had significantly higher biomass in the cover crop treatments (CC and CSCC), rather than CS, compared with that in the control (Table 2). This was consistent with the increasing trend of SOM content above (Table 1). Presumably, such differences resulted from the traits of organic materials. Rye cover crop favors the proliferation of AMF (García-González et al., 2018), which subsequently contributed to the increase in soil C storage due to their extensive mycelium and necromass in the presence of rye roots (Zhu and Michael Miller, 2003). Besides, the yearly input of root biomass from rye cover crop is nearly 44.8 g C m^{-2} (Austin et al., 2017), which should be sufficient to sustain a large AMF community.

Further exploration on bacterial community showed the greatest impact of CSCC on the bacterial compositions, compared to either CS or CC individually (Fig. 5 and Table 3). We ascribe this enhanced effect of CSCC to the fact that CSCC harbored both living and dead organic materials. In CSCC, some lineages that might prefer degrading those materials were enriched, such as Burkholderiales (Betaproteobacteria) and Sphingobacteriia (Bacteroidetes), while some K-strategists (oligotrophs) were constrained, such as Acidobacteria Gp6. Some Burkholderiales have the ability to decompose high molecular weight organic compounds (Gu et al., 2017), and Sphingobacteriia can utilize lignocellulosic material for growth (Yan et al., 2012), while Acidobacteria, in all likelihood, degrade ancient or recalcitrant SOM in nutrient-poor conditions (Wang et al., 2018). Since the bacterial structure of CSCC in this study separated from other treatments by $CHCl_3$ extracted lipids as revealed by RDA biplot (Fig. 6), and lipids could come from microbes and not just plants, it might be inferred that combining both treatments increases lipid abundance due to both plant and microbe inputs.

Despite the negligible shifts in the whole bacteria community (Fig. S4), CS increased the relative abundances of some lineages, such as Myxococcales (Deltaproteobacteria), but decreased that of Actinobacteria. Myxobacteria, ubiquitous in soil environments, are predators and can access nutrients from a broad spectrum of microorganisms (Thiery and Kaimer, 2020). Actinobacteria are a key bacterial group in the utilization of readily available C. The opposite trends of those groups

might imply an inverse relationship between them. It is noteworthy that the bacterial communities in stover-contained treatments (CS and CSCC) were significantly correlated to $CHCl_3$ -extracted components, i.e. proteins and lignin in the $CHCl_3$ extracts with CS, and lipids and unsaturated hydrocarbon in the $CHCl_3$ extracts with CSCC (Fig. 6). This feature suggested that in stover-contained treatments the bacteria preferred the mineral-associated SOM fractions, and further supported the necessity of using sequential extraction protocols, rather than water solvent only, to exhaustively depict the non-polymer SOM compounds. By contrast, no significant change in the bacterial community was observed for the single cover crop treatment, suggesting a different microbial selection in the corn stover soil.

Soil C stocks change slowly, usually on decadal time scales (Deng et al., 2016), but soil C characteristics can change more rapidly. For example, the proportion of C in the active versus passive pools can change after just a few years of altered management (Sprunger and Robertson, 2018). Here, we show that soil organic C composition and bacterial communities are also altered by changing inputs. Will this lead to increased total soil organic C? Perhaps. At our field site, the organic amendments lead to higher soil organic C on average (although not statistically significant), suggesting a trend toward higher C accumulation with cover crops and stover retention. In addition, the cover crop treatment increased the presence of aromatic compounds, and the stover increased the presence of lignin, which are both recalcitrant compounds. This trend may not be universal, however. The coarse texture and medium C concentration at our site are more likely to accumulate C than fine-textured soils or those with high clay and soil organic matter content (West and Six, 2007; Johnston et al., 2009; Sprunger and Robertson, 2018; Szymanski et al., 2019).

Similarly, it is not yet clear how shifts in microbial community composition will influence soil C accumulation. We observed the microbial community shift in parallel with soil C chemistry, possibly in a way that optimizes C consumption (see previous paragraphs in this section). As a result, these microbial community changes could lead to 1) increases in C mineralization that offset the additional C inputs, or 2) increased microbial biomass and necromass, which increases total soil organic C (Six et al., 2006; Cotrufo et al., 2015).

In this study we demonstrate the changes in SOM biochemical and microbial structure that occur from different types of SOM amendments. Although our results hint at mechanisms, we cannot yet link specific SOM structures to the soil bacterial community due to technical limitations. Nonetheless, this paper is an important first step in understanding mechanisms of SOM formation. Further research could examine the long-term fate of specific C compounds, perhaps via isotopically-labeled additions of these compounds, coupled with microbial community assessments. FT-ICR-MS is a useful tool for examining the degradation of organic C and for understanding the underlying mechanisms of soil C formation and retention.

5. Conclusions

This study provides insights regarding the effects of corn stover retention and cover crop on SOM compounds and the soil microbial community. Although there were negligible variations in total SOM content, total microbial biomass, and bacterial population due to these C amendment practices, significant changes were found for some SOM chemical compounds, AMF biomass and some bacterial lineages, with corn stover and cover crop showing different impacts on those parameters. Soil biosystems respond slowly to C management practices, with resultant soil changes occurring as a function of type of organic C addition, the microbiome, and the organic compounds added and their residual signatures.

Declaration of competing interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Support for this research was provided by the U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research (Awards DE-SC0018409 and DE-FC02-07ER64494), by the National Science Foundation Long-term Ecological Research Program (DEB 1637653) at the Kellogg Biological Station, by Michigan State University AgBioResearch, and by China Scholarship Council. A portion of the research was performed at the EMSL User Facility. We thank all people who provide help but were not listed as authors at EMSL.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.soilbio.2020.107986>.

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